Microsatellite polymorphisms in the epidermal growth factor receptor (*EGFR*) gene and the transforming growth factor-α (*TGFA*) gene and risk of oral cancer in Puerto Rico

Daehee Kang[a,b,*], Gloria Gridley[b], Wen-Yi Huang[b], Lawrence S. Engel[c,], Deborah M. Winn[d], Linda M. Brown[b], Eleuterio Bravo-Otero[e], Tianxia Wu[f,†], Scott R. Diehl[f,†] and Richard B. Hayes[b]

Objectives and methods **Risks of oral cancer related to a CA microsatellite repeat polymorphism in intron 1 of the epidermal growth factor receptor (*EGFR*) gene and a TaqI polymorphism in the transforming growth factor-α (*TGFA*) gene were evaluated in a population-based case–control study consisting of 157 cases and 149 controls recruited in Puerto Rico.**

Results **Carriers of ≥16 CA repeats in *EGFR* showed a 1.9-fold increased risk for oral cancer (OR=1.9, 95% CI=1.0–3.5). Risks also tended to increase with decreasing number of alleles with ≥16 CA repeats (*P* for trend=0.06). Our data suggested a non-significant reduction in risk for subjects heterozygous for the *TGFA* polymorphism (OR=0.6, 95% CI=0.2–1.3).**

Conclusions **The *EGFR*-associated risk appeared to be independent of tobacco and alcohol use and may be restricted primarily to subjects who consumed low amounts of fresh fruits and vegetables (OR=5.9, 95%CI: 2.3–15.2). These data implicate dietary and molecular targets for oral cancer prevention. *Pharmacogenetics and Genomics* 15:343–347 © 2005 Lippincott Williams & Wilkins.**

Pharmacogenetics and Genomics 2005, **15**:343–347

Keywords: oral cancer, *EGFR*, *TGFA*, Puerto Rico

[a]Department of Preventive Medicine, Seoul National University College of Medicine, Cancer Research Institute, SNUCM, Seoul, Korea, [b]Division of Cancer Epidemiology and Genetics, [c]Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, USA, [d]Division of Cancer Control and Population Sciences, National Cancer Institute, Bethesda, MD, USA, [e]University of Puerto Rico, San Juan, Puerto Rico and [f]Craniofacial Epidemiology and Genetics Branch, NIDCR, Bethesda, MD, USA.

Correspondence and requests for reprints to Daehee Kang, MD, PhD, Department of Preventive Medicine, Seoul National University College of Medicine, Cancer Research Institute, SNUCM, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799 Korea.
Tel: + 82 2 740 8326; fax: + 82 2 747 4830;
e-mail: dhkang@snu.ac.kr

Received 7 September 2004 **Accepted** 10 January 2005

Introduction

Epidermal growth factor receptor (EGFR), a transmembrane glycoprotein member of the erbB family of type-1-tyrosine kinases [1], plays a crucial role through downstream signaling pathways in cell cycle progression, survival, and proliferation [2]; however, EGFR is overexpressed in many tumor types and is associated with poorer clinical outcomes [3]. EGFR mRNA and protein and the EGFR ligand, transforming growth factor-α (TGFA), are found at increased levels in head and neck cancers and in histologically normal mucosa up to several centimeters away from the primary tumor site (but not in control normal mucosa) [4]. Aberrant overexpression of EGFR is also observed in premalignant oral leukoplakias [5], further suggesting the importance of the TGFA/EGFR signaling pathway in the early stages of oral carcinogenesis. Fewer CA repeats in an intron 1 polymorphism in *EGFR* are associated with greater in-vitro transcriptional activity [6] and a TaqI polymorphism in *TGFA* may be associated with the birth defect orofacial clefting [7–8], but the role of these genetic variants in oral cancer is unknown.

Oral cancer is more common in Puerto Rico (men 18.5/100 000, women 4.5/100 000) than among Hispanics in the USA 50 states (men 8.9/100 000, women 2.7/100 000) [9], with the greatest risks among heavy tobacco and alcohol users [10] and those who consume relatively low quantities of fresh fruits and vegetables [11]. In a population-based case–control study, we evaluated risks for oral cancer associated with the *EGFR* CA repeat and *TGFA* TaqI polymorphisms, in relation to previously identified oral cancer risk factors in this population.

Methods

Study subjects

These data are part of a population-based, case–control study conducted in Puerto Rico to investigate risk factors for oral cancer [10–12]. In brief, all Puerto Rican men and women, aged 21 to 79 years, diagnosed with newly incident histologically confirmed cancer of oral cavity and

Current address: *Division of Cancer Epidemiology and Genetics, National Cancer Institute, 6120 Executive Blvd., Bethesda,20892-7240 MD, USA
Tel: +301-451-5034; fax: +301-402-1819; e-mail: kangd@mail.nih.gov
†Center for Pharmacogenomics and Complex Disease Research, New Jersey Dental School, UMDNJ, Newark, New Jersey 07101, USA.

pharynx between December 1992 and February 1995, were included for study through the Puerto Rico Central Cancer Registry and island pathology laboratories. Population controls with no history of oral cancer were selected from residents of Puerto Rico. After exclusion of cases with cancer of the salivary gland because of small numbers (7%) and different etiology [13], 342 cases (70% of eligible) and 521 controls (83% of eligible) were interviewed. All subjects gave written informed consent to participate in the study. The study protocol was approved by the National Cancer Institute institutional review board.

Interview data

Detailed information on demographic characteristics, tobacco use (including tobacco type, age started, age stopped, total years and amount usually used), alcohol use (including beverage type, age started, age stopped, total years used and usual weekday and weekend consumption), usual adult diet, and other selected factors was collected by trained interviewers using a structured pre-tested questionnaire. Lifetime smoking was estimated from usual daily number of cigarettes smoked and total years of use. Cigarette smokers were persons who had smoked at least 100 cigarettes in their lifetime. Lifetime alcohol intake was estimated from weekly intake (combining weekday and weekend amounts) and total years of use. Persons were considered to be alcohol drinkers if they had at least 12 drinks of any kind of alcohol in their lifetime.

Diet was assessed through 104 food and beverage frequency questions asking the subjects to estimate usual consumption 'during most of their adult life'. The food-frequency questionnaire was designed specifically for this study population. Nutrient indices were computed by multiplying each subject's food-frequency responses by a matrix of standard portion size and nutrient density estimates, derived from USDA food composition tables for 375 food items, including several USDA food items specific for foods consumed in Puerto Rico.

Biological sample collection

A sub-set of 283 cases and 258 controls were selected to provide oral epithelial cell specimens based on their residence and date of interview. Detailed eligibility criteria were provided elsewhere [14]. We successfully obtained oral epithelial cell specimens and DNA samples for genotyping from 53% of eligible cases ($n = 149$) and 58% of controls ($n = 149$) [11].

Genotyping method

The *EGFR* microsatellite marker was fluorescently labeled and PCR reactions were performed in a total volume of 5 µl containing PCR buffer, 50 ng genomic DNA, 800 µM dinucleotide triphosphates (dNTPs), 0.25 µM. primers, and 0.25 U Taq polymerase. The reaction was incubated for 28 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. PCR products were size fractionated on 377 DNA Analyzers (trademark label) and genotypes were scored using GENESCAN and GENOTYPER computer programs [15–16]. The *TGFA* TaqI polymorphism PCR was performed in a total volume of 15 µl containing 40 ng of genomic DNA, 0.3 µM each primer, 200 µM dNTPs, PCR buffer, and 0.25 U Taq1 polymerase. The PCR products were resolved on 6% NuSieve gels to identify 117 and 113 bp products [7,17]. CA repeat polymorphisms of *EGFR* were assessed in 124 cases and 138 controls and *TGFA* polymorphisms were evaluated in 130 cases and 132 controls. Three cases did not have enough DNA for PCR amplification, 22 cases and 11 controls could not be assigned an EGFR genotype after repeated assay attempts and 15 cases and 17 controls failed for TGFA genotypes.

Statistical analysis

Odds ratios and 95% confidence intervals (CI) were calculated by unconditional logistic regression analysis, adjusted for age and sex. On the basis of the estimated relative risks for oral cancer [10], we categorized tobacco smokers into four groups (never, 1–10, 11–20, > 20 cigarettes/day). Alcohol users were categorized in tertiles of use (based on the distribution in the controls). For fresh fruits and vegetables, subjects were categorized in tertiles of consumption, again based on the distribution in controls. In multivariate analyses, categories were combined to increase the statistical power, with final categories designed to have similar numbers in each category. Thus, additional adjustment was carried out for smoking (never, 1–20, > 20 cigarettes/day), alcohol (drinks/week > 24 vs. ≤ 24), and fresh fruits and vegetable consumption (servings/day > 3.1 vs. ≤ 3.1). The statistical model was constructed by adjusting covariates identified as significant predictors from the initial univariate analyses. To test for trend, the exposure variables were treated as continuous in the model. The product variable between genotype and exposure risk factors [genotype $\times$ exposure] was included in the logistic model when evaluating the multiplicative interactive effect of selected genotypes and previously identified risk factors.

Results

The risk factor profiles for environmental exposures (i.e., tobacco, alcohol, and diet) among the sub-set of subjects with biological samples in this study were similar to those previously reported for the full case–control series (Table 1) [10–11]; risks increased as smoking and alcohol use increased and fresh fruits and vegetables consumption decreased. Self-reported ethnicity was comparable between cases (66% whites, 11% blacks,

Table 1 Selected characteristics of study subjects, Puerto Rico

Characteristic	Cases		Controls		OR*	95% CI
	n	%	*n*	%		
Smoking (cigs/days)						
Never	22	14.8	65	43.6	1.0	
≤ 10	21	14.1	40	26.8	1.4	0.7–3.0
11–20	42	28.2	26	17.4	4.3	2.1–8.9
>20	64	43.0	18	12.1	9.0	4.2–19.1
P trend					<0.001	
Alcohol (drinks/week)						
Never	13	8.7	41	27.5	1.0	
<6	11	7.4	36	24.2	1.1	0.4–3.1
6–24	19	12.8	36	24.2	2.9	1.0–8.7
>24	106	71.1	36	24.2	14.0	5.0–39.3
					<0.001	
Fresh fruits & vegetables (servings/day)						
>5	26	17.4	50	33.6	1.0	
3.1–5	37	24.8	49	32.9	1.6	0.8–3.1
<3.1	86	57.7	50	33.6	3.7	1.0–6.8
P trend					<0.001	

*Adjusted for age and sex.

Table 2 Allele frequencies of *EGFR* microsatellite polymorphisms

Polymorphisms	Cases		Controls		Caucasians*		African-Americans*	
	n	%	*n*	%	*n*	%	*n*	%
<16	10	4	13	5	14	4	9	5
16	93	38	89	32	158	43	70	42
17	23	9	30	11	21	6	26	15
18	44	18	47	17	59	16	11	7
19	10	4	14	5	7	2	15	9
20	46	19	67	24	77	21	24	14
>20	22	9	16	6	30	8	13	8
Total	248	100	276	100	366	100	168	100

*From Ref [15].

Table 3 Oral cancer risk by genetic polymorphism of *EGFR* microsatellite and *TGFA* TaqI

Genotype	Cases		Controls		OR*	95% CI
	n	%	*n*	%		
EGFR CA repeats						
both alleles >16	43	34.7	57	41.3	1.0	
one allele ≤ 16	59	47.6	60	43.5	1.8	0.9–3.5
both alleles ≤ 16	22	17.7	21	15.2	2.1	0.9–5.2
P for trend					0.06	
both alleles >16	43	34.7	57	41.3	1.0	
any alleles ≤ 16	81	65.3	81	58.7	1.9	1.0–3.5
TGFA						
C1/C1	117	89.3	107	81.1	1.0	
C1/C2	13	9.9	25	18.9	0.6	0.2–1.3
C2/C2	1	0.8	0	0	–	
C1/C1	117	89.3	107	81.1	1.0	
Any C2	14	10.7	25	18.9	0.7	0.4–1.4

*Adjusted for age (≤ 65, 66–71, ≥ 72), sex, smoking (never, 1–20, >20 cigarettes/day), alcohol (drinks/week >24 vs. ≤ 24), and fruit & vegetables consumption (servings/day >3.1 vs. ≤ 3.1).

23% others) and controls (70% whites, 7% blacks, 23% others). The frequency of the 13 CA dinucleotide repeat alleles in intron 1 of *EGFR* observed among our study subjects did not significantly differ between oral cancer cases and controls ($P = 0.57$). The frequencies in our subjects, respectively, for the most common alleles were: 16 CA dinucleotide repeat (118 bp fragment length): 38% in cases and 32% in control; 18 repeats (122 bp): 18% in cases and 17% in control; 20 repeats (126 bp): 19% in cases and 24% in control. The allele frequencies observed in this study are similar to a recent report among 183 Caucasians (43%, 16%, and 21%, respectively) and 84 African-Americans (42%, 7%, and 14%, respectively; Table 2) [15].

Although there were no overall differences in *EGFR* allele frequencies between oral cancer cases and controls, we conducted three exploratory analyses, each focusing on one of the three most common *EGFR* microsatellite alleles noted above, pooling the other 12 alleles into a single group, and adjusting for the established risk factors for oral cancer age, sex, smoking, alcohol drinking and consumption of fresh fruits and vegetables. This exploratory analysis most strongly suggested that the 16 CA repeat allele was associated with increased cancer risk. Because a functional study [6] suggests that expression of *EGFR* is related to the numbers of CA repeats, for primary analysis, we compared subjects having ≤ 16 CA repeats versus a pool of all alleles larger in size since alleles smaller than 16 CA repeats were rare (a total of 4% allele frequency in cases, 5% in controls, Table 2). We found that subjects having either one or two copies of an *EGFR* allele with ≤ 16 CA repeats showed 1.9-fold increased risk for oral cancer (OR = 1.9, 95% CI = 1.0–3.5), after adjustment for cigarette and alcohol use and fresh fruits and vegetable consumption. Risks also tended to increase with decreasing number of alleles with 16 CA repeats (P for trend = 0.06; Table 3).

The *TGFA* C2 allele was of low frequency in our study (6% in cases and 9% in controls, not significantly different) and similar to a previous report of 1319 unrelated individuals (7%) [17]. Heterozygotes for this allele showed a non-significant reduction in cancer risk (OR = 0.6, 95% CI = 0.2–1.3; Table 3).

The association between the *EGFR* short sequence repeat and oral cancer was stronger in heavy smokers (OR = 9.1, 95% CI = 2.3–35.7) and heavy alcohol users (OR = 11.9, 95% CI = 4.3–32.8), compared with never smokers or non/light drinkers with other genotypes. The patterns of risk were consistent with independent effects of the exposures and the gene variants (P for interaction = 0.99, for smoking, and P for interaction = 0.58, for

Table 4 Joint effect between *EGFR* microsatellite polymorphism and smoking, alcohol and fruit/vegetables consumption on oral cancer (cases/controls)

	Smoking			Alcohol		Fresh fruits and vegetables	
	Never	≤ 20 cigs/day	>20 cigs/day	≤ 24/wk	>24/wk	>3.1/day	≤ 3.1/day
EGFR CA repeats							
both alleles >16	1.0 (5/26)	3.8 (1.4–10.6) (15/23)	4.8 (1.6–15.0) (23/8)	1.0 (10/41)	7.6 (3.3–17.6) (33/16)	1.0 (15/39)	2.2 (1.0–4.6) (28/18)
any Alleles ≤ 16	2.0 (0.6–6.9) (14/33)	6.8 (2.1–21.7) (35/40)	9.1 (2.3–35.7) (32/8)	2.2 (0.9–5.4) (24/63)	11.9 (4.3–32.8) (57/18)	1.2 (0.5–3.0) (38/51)	5.9 (2.3–15.2) (43/50)
OR (95% CI) in each subgroup	2.0 (0.5–7.0)	1.7 (0.7–4.3)	2.0 (0.5–8.4)	2.2 (0.9–5.4)	1.6 (0.6–3.8)	1.2 (0.5–3.0)	2.6 (1.1–6.3)
P for interaction	0.99			0.58		0.21	

*Adjusted for age ≤ 65, 66–71, ≥ 72), sex, and other variables; smoking (never, 1–20, >20 cigs/day), alcohol (drinks/wk >24 vs. ≤ 24), and fruit & vegetables consumption (servings/day >3.1 vs. ≤ 3.1).

alcohol; Table 4). Carriers of alleles with ≥ 16 CA repeats in *EGFR* who had low fresh fruit and vegetable intake were at excess risk for oral cancer (OR = 5.9, 95%CI: 2.3–15.2) compared to high consumers of fresh fruits and vegetables with other genotypes (Table 4). There were no significant interactive effects observed between *TGFA* variants and other known risk factors (data not shown).

Discussion

Our study in Puerto Rico is the first to suggest that risks for oral cancer may be greater in carriers of short-sequence CA dinucleotide repeats in intron 1 of *EGFR*. This is consistent with in-vitro studies showing that alleles with 16 CA repeats are associated with increased EGFR expression [6] and human studies showing that increased expression is related to oral carcinogenesis [5,18] and cancers at other sites [1,19–20]. The TaqI variant in *TGFA*, for which there is only indirect evidence of functional effects [7–8], showed no significant relation to oral cancer risk. However, since the frequency of the less common *TGFA* allele is very low, our power to detect an association is limited.

Risks are greatest for the subset of subjects with fewer CA repeats in *EGFR* who were heavy smokers and alcohol drinkers; however, the findings of no significant multiplicative interaction between smoking, alcohol and *EGFR* genotype indicate that tobacco and alcohol-associated risks may act independently of *EGFR* genotype status. Tobacco–alcohol oral carcinogenesis probably operates through genotoxic mechanisms [21], independent of EGFR-regulated cell-cycle control and proliferation. For public health concerns, however, these independent genetic effects add substantially to the high predictive risks for oral cancer in people with these high-risk behaviors.

Although the relative protection of dietary factors appears more modest than the tobacco- and alcohol-associated increases in risk, there is much epidemiologic support for our finding that fresh fruits and vegetables provide protection from oral cancer [22]. Our study showed increased risks for oral cancer only in low users of fresh fruits and vegetables and in this group fewer *EGFR* CA repeats tended to be related to relatively greater risks. In contrast, no impact of *EGFR* genotype on oral cancer risk was found in subjects consuming greater amounts of fresh fruits and vegetables. Although this contrast is intriguing, we note that the formal test for interaction was not significant ($P = 0.21$, Table 4) and the finding should be viewed with caution pending replication in an independent sample.

Retinoids and related Vitamin A analogs are anticarcinogenic for oral and respiratory epithelia [23–25], possibly through *EGFR* down-regulation [26]; however, the chemically similar beta-carotene, found in fruits and vegetables, does not appear to offer such protection [26,27]. In our study we also ruled out dietary folate as a protective source [11]. Other components of fresh fruits and vegetables may impact on the *EGFR* signaling pathway.

Our results for *EGFR* could be an artifact due to 'population stratification', if (a) the 'at-risk' genotypes occurred more frequently in a particular ethnic sub-group of the Puerto Rican population and (b) this same ethnic group was, for other reasons, at greater risk for oral cancer (e.g., were consumers of greater amounts of tobacco and alcohol). In our study, self-reported ethnicity did not differ substantially between cases (66% whites, 11% blacks, 23% others) and controls (70% whites, 7% blacks, 23% others) suggesting that ethnicity, per se, is not an important risk factor for oral cancer in Puerto Rico. Also, for our study the allele frequency pattern did not differ for self-reported whites and blacks. And further, from a US study no differences in allele frequency were observed for whites and African-Americans. We found no evidence that ethnicity is a risk factor in Puerto Rico

or that *EGFR* variability is associated with ethnicity, the two criteria necessary for false association due to population stratification.

In conclusion, this study of polymorphic variants in cell-cycle-related *EGFR* and *TGFA* genes suggested that short sequence CA repeats in *EGFR*, associated with increased expression, may be related to increased risk for oral cancer and that these effects may be most prominent in subjects with diets deficient in protective agents found in fresh fruits and vegetables. However, larger epidemiologic studies and expanded mechanistic investigations will be needed to establish these relationships.

Acknowledgements

We thank Glenn Miller, Phuong-Tu Le, Luanne Lukes and Cui-e Sun for performing genotyping assays.

References

1 Kari C, Chan TO, de Quadros MR, Rodeck U. Targeting the epidermal growth factor receptor in cancer: apoptosis takes center stages. *Cancer Res* 2003; **63**:1–5.
2 Boulougouris P, Elder J. Epidermal growth factor receptor structure, regulation, mitogenic signalling and effects of activation. *Anticancer Res* 2001; **21**:2769–2775.
3 Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol* 2003; **21**:2787–2799.
4 Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res* 1993; **53**: 3579–3584.
5 Srinivasan M, Jewell SD. Evaluation of TGF-alpha and EGFR expression in oral leukoplakia and oral submucous fibrosis by quantitative immunohistochemistry. *Oncology* 2001; **61**:284–292.
6 Gebhardt F, Zanker KS, Brandt B. Modulation of epidermal growth factor receptor gene transcription by a polymorphic dinucleotide repeat in intron 1. *J Biol Chem* 1999; **274**:13176–13180.
7 Machida J, Yoshiura K, Funkhauser CD, Natsume N, Kawai T, Murray JC. Transforming growth factor-alpha (TGFA): genomic structure, boundary sequences and mutation analysis in nonsyndromic cleft lip/palate and cleft palate only. *Genomics* 1999; **61**:237–242.
8 Vieira AR, Orioli IM. Candidate genes for nonsyndromic cleft lip and palate. *J Dent Child* 2001; **68**:272–279.
9 Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999; **80**:827–841.
10 Hayes RB, Bravo-Otero E, Kleinman DV, Brown LM, Fraumeni JF Jr, Harty LC, Winn DM. Tobacco and alcohol use and oral cancer in Puerto Rico. *Cancer Causes Control* 1999; **10**:27–33.
11 Weinstein SJ, Gridley G, Harty LC, Diehl SR, Brown LM, Winn DM, Bravo-Otero E, Hayes RB. Folate intake, serum homocysteine and methylenetetrahydrofolate reductase (MTHFR) C677T genotype are not associated with oral cancer risk in Puerto Rico. *J Nutr* 2002; **132**: 762–767.
12 Harty LC, Caporaso NE, Hayes RB, Winn DM, Bravo-Otero E, Blot WJ, *et al.* Alcohol dehydrogenase 3 genotype and risk of oral cavity and pharyngeal cancers. *J Natl Cancer Inst* 1997; **89**:1698–1705.
13 Horn-Ross PL, Morrow M, Ljung BM. Menstrual and reproductive factors for salivary gland cancer risk in women. *Epidemiology* 1999; **10**:528–530.
14 Harty LC, Shields PG, Winn DM, Caporaso NE, Hayes RB. Self-collection of oral epithelial cell DNA under instruction from epidemiologic interviewers. *Am J Epidemiol* 2000; **151**:199–205.
15 Liu W, Innocenti F, Chen P, Das S, Cook EH Jr, Ratain MJ. Interethnic difference in the allelic distribution of human epidermal growth factor receptor intron 1 polymorphism. *Clin Cancer Res* 2003; **9**:1009–1012.
16 Ziegle JS, Su Y, Corcoran KP, Nie L, Mayrand PE, Hoff LB, *et al.* Application of automated DNA sizing technology for genotyping microsatellite loci. *Genomics* 1992; **14**:1026–1031.
17 Basart AM, Qian JF, May E, Murray JC. A PCR method for detecting polymorphism in the TGFA gene. *Hum Mol Genet* 1994; **3**:678.
18 Song JI, Grandis JR. STAT signaling in head and neck cancer. *Oncogene* 2000; **19**:2489–2495.
19 Franklin WA, Veve R, Hirsch FR, Helfrich BA, Bunn PA Jr. Epidermal growth factor receptor family in lung cancer and premalignancy. *Semin Oncol* 2002; **29**(**1 Suppl 4**):3–14.
20 Ibrahim SO, Vasstrand EN, Liavaag PG, Johannessen AC, Lilehaug JR. Expression of c-erbB proto-oncogene family members in squamous cell carcinoma of the head and neck. *Anticancer Res* 1997; **17**:4539–4546.
21 Wight AJ, Ogden GR. Possible mechanisms by which alcohol may influence the development of oral cancer – a review. *Oral Oncol* 1998; **34**:441–447.
22 Winn DM. Diet and nutrition in the etiology of oral cancer. *Am J Clin Nutr* 1995; **61**:437S–445S.
23 Sankaranarayanan R, Mathew B, Varghese C, Sudhakaran PR, Menon V, Jayadeep A, *et al.* Chemoprevention of oral leukoplakia with vitamin A and beta carotene: an assessment. *Oral Oncol* 1997; **33**:231–236.
24 Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, *et al.* Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1990; **323**:795–801.
25 Pastorino U, Infante M, Maioli M, Chiesa G, Buyse M, Firket P, *et al.* Adjuvant treatment of stage I lung cancer with high-dose vitamin. *Am J Clin Oncol* 1993; **11**:1216–1222.
26 Lonardo F, Dragnev KH, Freemantle SJ, Ma Y, Memoli N, Sekula D, *et al.* Evidence for the epidermal growth factor receptor as a target for lung cancer prevention. *Clin Cancer Res* 2002; **8**:54–60.
27 Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, *et al.* Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med* 1996; **334**:1145–1149.